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A nylon membrane based amperometric biosensor for polyphenol determination

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ABSTRACT

A nylon membrane based amperometric biosensor employing banana fruit polyphenol oxidase (PPO) is presented for polyphenol detection. Nylon membrane was first activated and then coupled with chitosan. PPO was covalently attached to this membrane through glutaraldehyde coupling. The membrane bioconjugate was characterized by scanning electron microscopy (SEM) and Fourier Transform Infrared (FTIR) study and then mounted onto Au electrode using parafilm to construct a working electrode. Once assembled along with Ag/AgCl as reference and Pt as auxiliary electrode, the biosensor gave optimum response within 15 s at pH 7.5 and 30 °C, when polarized at +0.4 V. The response (in mA) was directly proportional to polyphenol concentration in the range 0.2–400 μM. The lower detection limit of the biosensor was 0.2 μM. The biosensor was employed for determination of polyphenols in tea, beverages and water samples. The enzyme electrode showed 25% decrease in initial activity after 150 reuses over 6 months, when stored at 4 °C.

1. Introduction

Polyphenolic compounds are natural constituents of fruits and vegetables [1] and thus greatly influence food quality and storage as well as human health [2]. However, the effect of polyphenols on human health is contradictory. Some polyphenols play role in prevention of cardiovascular diseases, cancer and diabetes [3–6], while certain members of polyphenol family are considered as endocrine disrupting compounds [7–9]. Consequently, it is of great significance to determine polyphenolic compounds to study food quality, healthcare and pollution monitoring [10,11]. The available detection methods for polyphenols include mainly chromatographic techniques such as high-performance liquid chromatography (HPLC) and spectrometry [1]. However, these methods are cumbersome and expensive as they require several operations including pretreatment of the sample and costly equipments, which make them time-consuming as well [9,12].

Immobilized enzyme based biosensors present a fast and reliable alternative owing to their simplicity, specificity, fast response and reusability, which make them cost effective [13]. Numerous biosensors have been proposed for detection of polyphenolic compounds employing polyphenol oxidase (PPO). PPO, also known as catecholoxidase or tyrosinase, is widely distributed in plant kingdom [14]. It is a copper containing metalloprotein, which is known to catalyse two types of reactions using oxygen: (i) the *o*-hydroxylation of monophenols such as tyrosine and *o*-cresol to *o*-diphenols and (ii) the dehydrogenation of *o*-dihydroxyphenols such as catechol and L-3,4-dihydroxy phenylalanine (L-DOPA) to *o*-diquinones [15–17]. Various matrices have been used to immobilize PPO such as polyethersulphone membrane [10], laponite clay coating [18], gelatin [19], carbon paste modified electrodes [20], cobalt (II) phthalocyanine (CoPc) modified cellulose-graphite composite on a polycarbonate support [21], eggshell membrane [22], ZnO nanorod clusters/nanocrystalline diamond electrode [23], 2,2′-bipyridine chloro (p-cymene) ruthenium (II)]chloride mediator complex and 1,2-diamino benzene (DAB) [24], Fe_3O_4 nanoparticle-chitosan bionanocomposite film [25], polyvinyl chloride membrane [26], menthyl monomer (MM) with pyrrole [27], polyaniline [28], 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H* pyrrole [29], graphite screen printed electrodes modified with ferrocene [30] and thionine modified carbon paste electrode [9]. Compared to commercial enzyme, PPO purified from a plant source is economical and more stable and thus more suitable for construction of polyphenol biosensors. PPO has been purified and characterized from banana fruit as early as 1981 [31]. It has also been extracted from the leaves and stems of the banana for the production of L-DOPA [32].

Nylon is an inherently hydrophilic membrane, which is compatible with aqueous and alcoholic solutions and solvents. It is made of repeating units linked by peptide bonds (or amide bonds) and frequently referred to as polyamide. It is cationic and maintains its positive charge over a wide pH range. Nylon membrane also offers narrow pore size distribution and good mechanical rigidity. To improve the hydrophilicity and to increase the number of reac-

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tive sites (i.e. primary amino groups), the nylon membrane was activated by epichlorohydrin and then coupled with chitosan [33]. The present study, describes for the first time, the covalent immobilization of banana fruit PPO on chitosan coated nylon membrane through glutaraldehyde coupling and its application in construction of a polyphenol biosensor.

2. Materials and methods

2.1. Materials

L-DOPA from HiMedia (Mumbai, India), glutaraldehyde, polyvinyl pyrrolidone (PVP), epichlorohydrin and chitosan from SRL (Mumbai, India) and nylon membrane from Amresco, Ohio, US were used. All other chemicals were of analytical grade. Tea leaves (Brand: "Tata tea" manufactured by Tata global Beverages Limited, Kirloskar Business park, Block-C, Hebbal, Bengaluru, India. "Darjeeling" manufactured by Twining Private Limited, Kolkata, West Bengal, India. "Red label" and "Taj Mahal" manufactured by Brooke Bond, Hindustan Unilever Limited, (HUL), Backbay Reclamation, Mumbai, India) and alcoholic beverages of various commercial brands ("Vodka" manufactured by Radika Khaitan Limited, Badhali, Ambala, India. "Royal challenge" manufactured by United Spirits Limited Unit Merrut, Cant U.P, India. "Kingfisher Beer" manufactured by Millennium Beer, Industries Limited, Dharuhera, Rewari, Haryana, India. "Whisky" manufactured by Bagpiper, Mathura Road, Palwal, Haryana, India.) (All 2010 products) were purchased from local market. Water samples were collected from different sources like tap water, well and canal samples from a nearby rural area of Rohtak. L-DOPA was added into freshly prepared 0.1 M sodium phosphate buffer pH 7.0 to give a final concentration of 10 mM, for assay of PPO.

2.2. Extraction of PPO from banana fruit

Pulp of fresh ripened banana fruit (25 g) was homogenized with 100 mL 0.1 M sodium phosphate buffer (pH 7.0) containing 2.5 g of PVP-360 as stabilizer, in a liquefier for 2 min at 4 °C. The suspension was filtered through a Whatman filter paper no. 1 on ice and centrifuged at 25,000 × *g* for 30 min at 4 °C. The supernatant (crude enzyme) was collected and stored at 4 °C until used [33].

2.3. Assay of PPO

The assay of PPO in crude extract was carried out by measuring the change in absorbance (ΔA) at 475 nm in a UV visible spectrophotometer (Shimadzu, Model 1700) due to conversion of L-DOPA into dopachrome. The reaction mixture containing 100 μL of crude enzyme and 3.0 mL of 10 mM L-DOPA solution in 0.1 M sodium phosphate buffer (pH 7.0) was incubated at 25 °C [34]. Under the specified assay conditions, one enzyme unit is defined as:

$$\text{Unit activity} = \frac{\Delta A_{475}/\text{min} \times \text{total vol. of reation mixture}}{E \times \text{vol. of enzyme}}$$

[E = 3.6 (extinction coefficient of L-DOPA); total volume = 3.1; enzyme volume = 0.1 mL]

The protein concentration in PPO preparation was determined by Lowry method [35], using bovine serum albumin as standard.

2.4. Activation/pretreatment of nylon membrane

Chitosan-coated nylon membrane, which possesses a large number of reactive groups $-CH_2OH$ and $-NH_2$, was prepared by coupling chitosan onto the nylon membrane. A rectangular piece of nylon membrane (4 cm × 4 cm) was soaked into 1 M HCl (10 mL) and stirred for 24 h at room temperature. After partial hydrolysis of amide bonds, the membrane was put into a 20% epichlorohydrin solution, adding NaOH to adjust the pH of the solution to pH 11, and then, the resulting mixture was stirred for 10 h at 60 °C. The activated membrane was shaken in 10 mL chitosan solution (prepared by dissolving 0.15 g chitosan in 10 mL 1% acetic acid solution) for 1 h at room temperature. The chitosan solution was sucked and then membrane was incubated in an oven at 80 °C for 7 h. Non-covalently bound chitosan was removed by washing the membrane with 1% acetic acid solution and deionized water [33].

2.5. Immobilization of PPO onto the chitosan coated nylon membrane

The chitosan coupled membrane was activated by dipping it into a 2.5% glutaraldehyde in 0.02 M sodium phosphate buffer, pH 7.0 and kept it for 2 h at room temperature. The membrane was removed and washed thoroughly in 0.02 M sodium phosphate buffer, pH 7.0. The activated membrane was dipped into 1 mL of enzyme solution containing 13 enzymatic U and kept overnight at 4 °C for covalent immobilization of enzyme. The nylon membrane containing immobilized enzyme was washed in the reaction buffer and tested for enzyme activity.

2.6. Scanning electron microscopy (SEM) of nylon membrane

The nylon membrane (1 cm × 1 cm) with and without enzyme was subjected to scanning electron microscopy (SEM) at Chemistry Dept. of MDU, Rohtak, to confirm the immobilization.

2.7. Fourier Transform Infrared (FT-IR) study of nylon membrane

The nylon membrane (1 cm × 1 cm) before and after immobilization of enzyme was placed between two KBr disks for the mid-IR characterization, using a FTIR spectrometer (mode iS10, Thermoelectron, USA) instrument.

2.8. Construction and response measurement of amperometric polyphenol biosensor

An amperometric polyphenol biosensor was constructed by mounting PPO-nylon membrane biocomposite onto Au wire electrode (1.5 cm × 0.05 cm) with a parafilm and connecting this working electrode along with a silver/silver chloride (Ag/AgCl) reference electrode and Pt as auxiliary electrode to a three-electrode electrochemical cell system employing potentiostat/galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785). To measure the response of the three electrode system, it was immersed into a beaker containing mixture of 2.9 mL of 0.1 M phosphate-EDTA buffer, pH 7.0 and 0.1 mL L-DOPA (10 mM). The electrode was polarized by applying different potential in the range +0.1 to +0.6 V against Ag/AgCl. The potential, at which the maximum current generated, was noted using the linear sweep method. The following electrochemical reactions occurred during measurement:

$$\text{L-DOPA} + 1/2O_2 \xrightarrow{\text{PPO}} \text{Benzoquinone derivative} + H_2O$$

In terms of electron transfer, the reaction can be written as:

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

The depletion of oxygen at the electrode caused by electrochemical reaction also involves consumption of electrons, resulting in an electrochemical signal, which was directly proportional to the concentration of polyphenols in the sample.

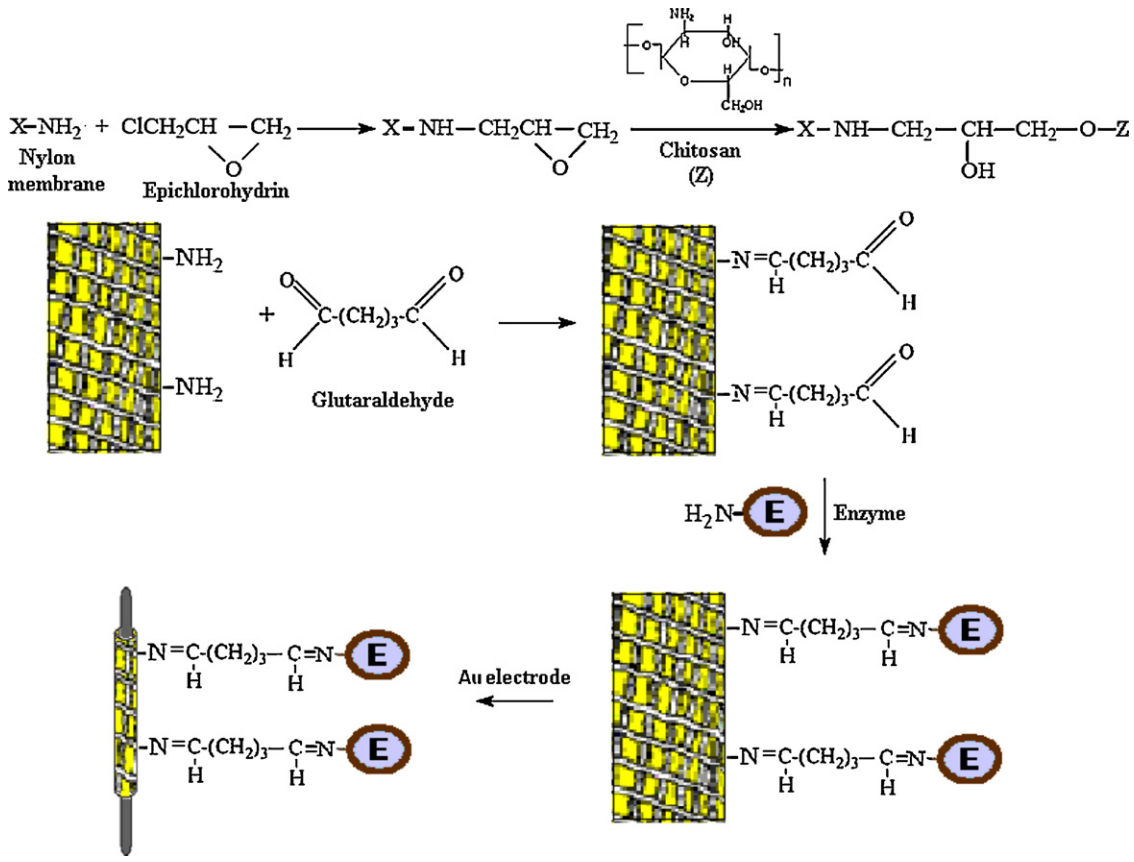

Fig. 1. Schematic representation of the mechanism of immobilization of PPO on nylon membrane using chitosan-glutaraldehyde cross-linking.

2.9. Optimization study

Various kinetic properties of the nylon membrane bound enzyme/enzyme electrode such as optimum pH, incubation temperature, response time and effect of substrate (L-DOPA) concentration were studied amperometrically to determine the optimum working conditions of the biosensor.

2.10. Amperometric determination of polyphenols in tea, alcoholic beverages and water samples

The biosensor was employed for determination of polyphenols in five commercial brands of tea leaves, alcoholic beverages and four water samples. To prepare the tea leaf extract, 5 g of dried tea leaves were boiled in 20 mL of distilled water for 5 min and the extract was filtered through a sieve. Alcoholic beverages and water samples were used as such. Polyphenol level in tea leaf extract, alcoholic beverages and water samples was determined by the biosensor in the same manner as described above for its response measurement under its optimal working conditions except that L-DOPA was replaced by the sample. The current (mA) was recorded and the amount of polyphenols was extrapolated from standard curve between L-DOPA concentrations and current (mA) prepared under optimal working conditions. To study the accuracy of the biosensor, the polyphenol level in tea samples was determined by standard Folin-Ciocalteu (FC) spectrophotometric method and by the present method and values obtained were correlated using regression equation. The FC method was carried out according to

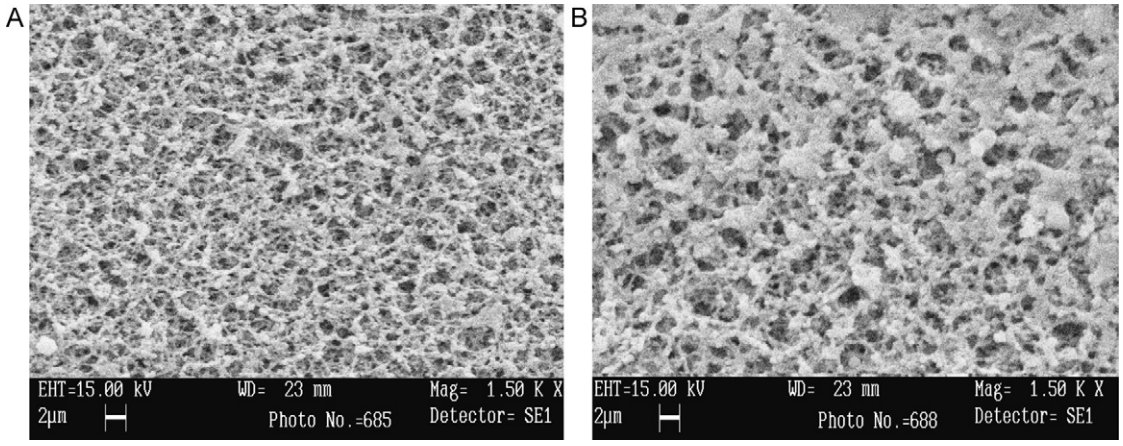

Fig. 2. Scanning electron micrographs of chemically modified nylon membrane without immobilized PPO (a) and with (b) immobilized PPO.

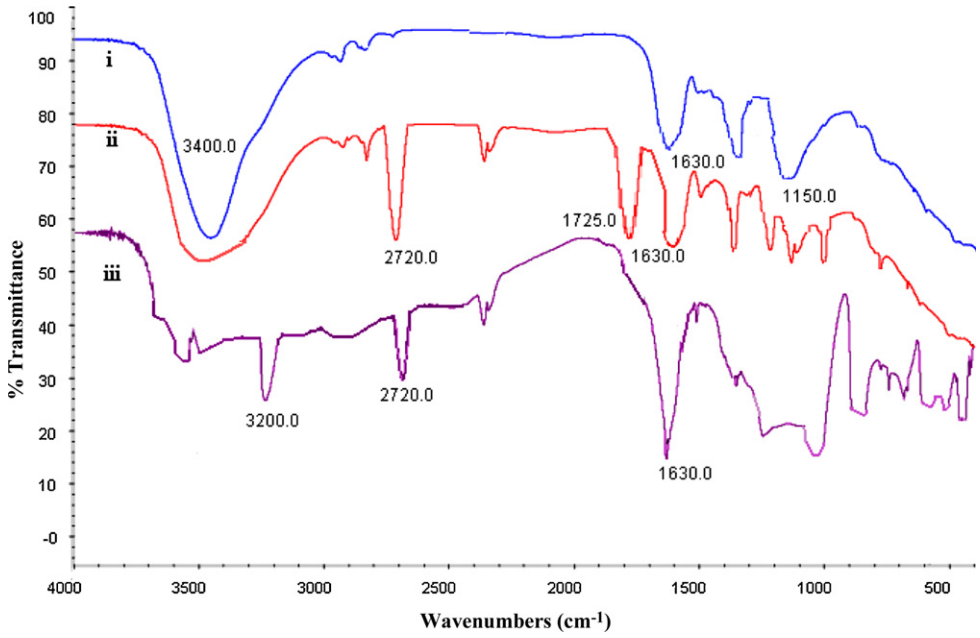

Fig. 3. FT-IR spectra of nylon membrane (i), nylon membrane with glutaraldehyde (ii) and nylon membrane with glutaraldehyde and enzyme (Curve iii).

Table 1
A comparison of various amperometric polyphenol biosensors.

Property	Kiralp et al. [27]	Tembe et al. [22]	Tan et al. [28]	Chawla et al. [26]	Present
Support for immobilization	Menthyl monomer (MM) with pyrrole	Eggshell membrane	Polyaniline membrane	Polyvinyl chloride membrane	Nylon membrane
Method of immobilization	Entrapment	Cross-linking	Entrapment	BSA-glutaraldehyde cross-linking	Covalent coupling
Optimum pH	9.0	5.0–6.5	6.5	7.5	7.5
Optimum temperature (°C)	40	30	45	35	35
Mode of measurement	Current	Current	Current	Current	Current
Response time (s)	NR	60	NR	30	15
Limit of detection (M)	NR	2.5×10^{-5}	1.25×10^{-6}	7.5×10^{-7}	0.2×10^{-6}
Linear range (M)	NR	5×10^{-5} to 25×10^{-5}	1.25×10^{-6} to 150×10^{-6}	1.25×10^{-6} to 1×10^{-5}	$0.2–400 \times 10^{-6}$
Storage life at 4 °C (days)	40	180	90	60	180

NR = not reported.

the procedure [36]. One mL of tea sample was transferred to a 15 mL test tube and 9 mL of a mixture of 50% ethanol and 50 mM H_3PO_4 was added to extract the polyphenols in the sample. The test tube was shaken overnight, at 5 °C and then centrifuged at 8000 × *g* for 10 min. 40 μL of tea extract was pipetted in a cuvette and 60 μL of 5% ethanol was added. 200 μL of FC reagent was then added, followed by 2 mL 2% Na_2CO_3 and 1 mL deionized H_2O. The content of the cuvette was agitated and then incubated for 2 h. Total polyphenol determination according to FC [36] is based on reduction of yellow FC reagent in basic environment to blue pigments by polyphenols. The samples were run on a spectrophotometer at 765 nm. Each sample was measured thrice.

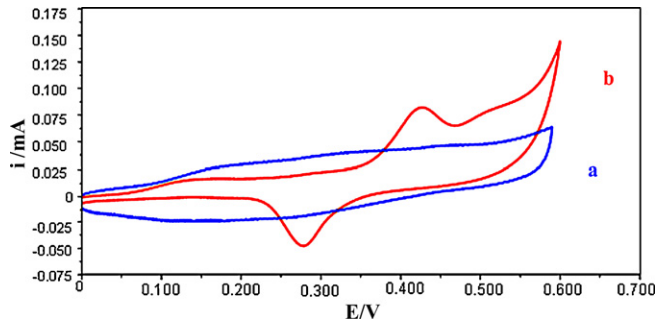

Fig. 4. Cyclic voltammograms of modified membrane electrode without immobilized enzyme (a) or with immobilized enzyme (b) in 0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 M KCl and 10 mM L-DOPA.

2.11. Interference study

The signal for a fixed concentration of substrate was finally compared with the signal obtained in the presence of the two interfering species, viz. ascorbic acid and uric acid each at 0.1 mM.

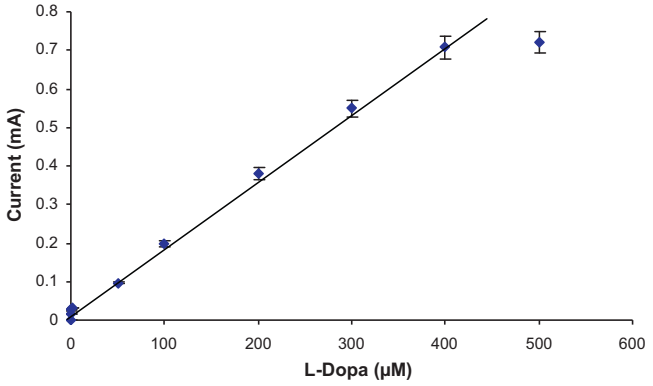

Fig. 5. Standard curve of L-DOPA concentration on response of polyphenol biosensor based on nylon bound polyphenol oxidase at +0.4 V vs. Ag/AgCl, pH 7.5 and 35 °C.

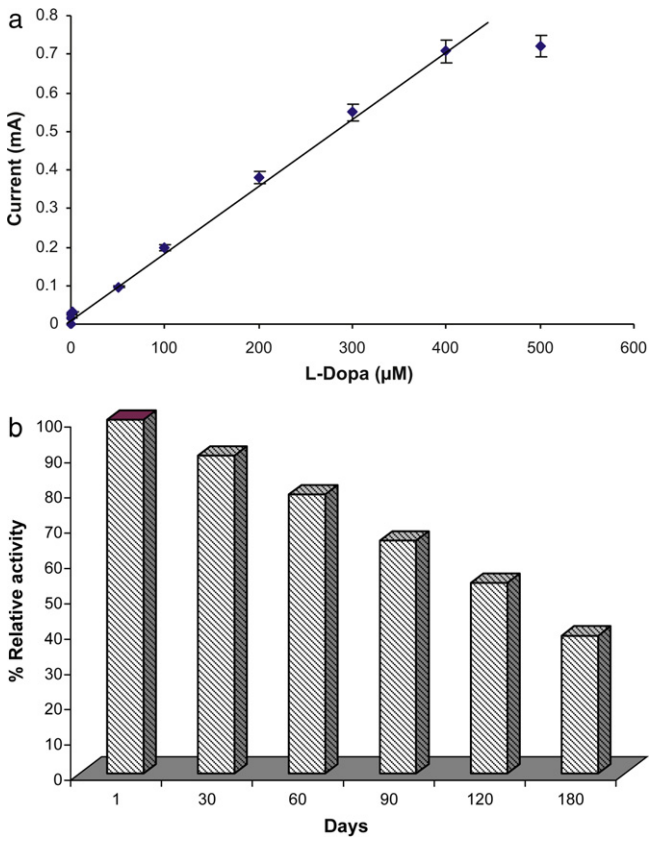

Fig. 6. (a) Correlation between tea polyphenol values determined by spectrophotometric method and present method. (b) Storage stability of polyphenol oxidase electrode in a 0.1 M sodium phosphate buffer pH 7.0 at 4 °C.

2.12. Reusability and storage of electrode

To reuse the enzyme electrode, it was washed three to four times with the reaction buffer (0.1 M sodium phosphate buffer pH 7.0), dried in between folds of tissue paper before its use in following assay. The enzyme electrode was stored in the same buffer at 4 °C, when not in use.

Table 2
Polyphenol level in different brand of tea leaves, alcoholic beverages and water samples as measured by polyphenol biosensor based on nylon membrane bound banana fruit polyphenol oxidase.

Brand	Polyphenol level (μM)Mean ± SE*
1. Tea leaves	
(a) Tata tea	150.7 ± 0.2
(b) Red label	160.1 ± 0.3
(c) Darjeeling	150.9 ± 0.7
(d) Taj	150.3 ± 0.6
(e) Local tea	180.1 ± 0.2
2. Alcoholic beverages	
(a) Vodka	140.6 ± 0.6
(b) Royal Challenge	150.2 ± 0.2
(c) Kingfisher Beer	140.8 ± 0.4
(d) Whisky	150.6 ± 0.7
(e) Local	170.4 ± 0.5
3. Water	
(a) Well	1.6 ± 0.6
(b) Canal	1.5 ± 0.5
(c) Tap	1.4 ± 0.7
(d) Distilled	0.0

Standard error = standard deviation/$n^{1/2}$.
* SE = standard error.

3. Results and discussion

3.1. Immobilization of PPO onto nylon membrane

Banana fruit PPO was immobilized onto chitosan coated nylon membrane through glutaraldehyde coupling with a 66.6% retention of initial activity of free enzyme and 4.44 mg/cm^2 conjugation yield. One –CHO group of glutaraldehyde was linked to –NH_2 group of enzyme, while another –CHO group of glutaraldehyde was bound to –NH_2 group of chitosan coupled on nylon membrane. This provided more stable enzyme membrane conjugate (Fig. 1). The SEM image of membrane with and without enzyme showed a change in surface morphology of membrane after immobilization (appearance of sporadic globules on enzyme membrane conjugate) confirming the possible PPO immobilization (Fig. 2).

Fig. 3 shows Fourier Transform Infrared (FT-IR) spectra for nylon membrane alone (Curve i), nylon membrane with glutaraldehyde (Curve ii) and nylon membrane with glutaraldehyde and enzyme (Curve iii). Curve i shows a broad band at 3400 cm^{-1}, usually assigned to the –N–H bending vibration in primary amine, is obviously enhanced. Curve ii shows the absorbance at 1725 cm^{-1} related to –C=O bond of aldehyde, while the peak at 2720 cm^{-1} also indicated the presence of free aldehyde group, which reveals that glutaraldehyde gets coupled with membrane. Curve iii shows a peak of –N=C– bond at 1630 cm^{-1}, while the absence of peak at 1725 cm^{-1} confirms that –C=O– group of glutaraldehyde got combined with –NH_2 groups on surface of enzyme to form –N=C bond. It also revealed that there was no free aldehyde group of glutaraldehyde, as it got cross-linked with enzyme, while appearance of peak at 3200 cm^{-1} revealed the presence of free alcoholic group of enzyme (PPO). These studies confirmed that the enzyme was immobilized through glutaraldehyde coupling on the surface of chitosan coated nylon membrane.

3.2. Cyclic voltammetry study

The membrane electrode without and with immobilized enzyme was characterized by cyclic voltammetry. Fig. 4 shows a typical cyclic voltammogram obtained with the membrane electrode in 0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 M KCl by addition of 0.1 mL L-DOPA solution (10 mM). An increase in the oxidation peak current at +0.411 V vs. Ag/AgCl was observed, when substrate was added to the solution.

3.3. Optimization of PPO biosensor

The sensor showed optimum response within 15 s at pH 7.5 and 35 °C. The effect of L-DOPA on the biosensor response was studied in the concentration range 0.2–400 μM (Fig. 5). There was a linear relationship between biosensor response/immobilized PPO activity and L-DOPA concentration up to 400 μM, after which it was constant. Michaelis constant (Km) for L-DOPA was 25 μM. Maximum current (I_{max}) was 1.025 μA min^{-1} mL^{-1}. The change in kinetics of PPO after immobilization might be due to many factors like chemical modification of the enzyme, conformational changes, steric effects, partitioning effects due to electrostatic or hydrophobic interactions between matrix and the low molecular weight species in solution causing modification of enzyme microenvironment and mass transfer or bulk diffusional effects arising from diffusional resistance to the transport of substrate from bulk solution to the catalytic sites as well as diffusion of reaction products back to the bulk solution [37]. A comparison of kinetic and analytic properties of the present biosensor with those of earlier amperometric polyphenol biosensors is summarized in Table 1.

3.4. Evaluation of the biosensor

The limit of detection of the present biosensor was calculated as the lowest quantity of L-DOPA required to give a signal to a background (blank) +3 times SD of blank and found to be 0.2 μM. The recovery of added L-DOPA in tea, alcoholic beverages and water samples (1 mg L^{-1} and 2 mg L^{-1}) were (94.0 ± 2.7)% & (96.0 ± 3.3)%, (98.0 ± 1.7)% & (99.0 ± 2.3)% and (90.2 ± 2.9)% and (96.0 ± 1.3)% (mean ± SD; n = 6) respectively. The intra and inter assay coefficient of variation (CVs) for polyphenol determination in tea samples by the present biosensor were <7.1% and <6.7%, respectively. These results indicate a better analytical performance of the present biosensor compared to earlier biosensors [26]. In order to know the accuracy of present methods, the levels of polyphenol in samples were determined by spectrophotometric method (x) and compared with those obtained by present method (y). The levels of polyphenol obtained by the present method matched with the standard one and showed a good correlation (r = 0.994), being the regression equation $y = 0.9832x + 0.0949$ (Fig. 6a).

No noticeable changes in current were detected in the presence of uric acid (0.1 mM), however ascorbic acid (0.1 mM) caused 15% inhibition at the present operating potential.

The enzyme electrode was reused 150-times over a period of 180 days, when stored at 4 °C in 50% glycerol solution (v/v) (Fig. 6b).

3.5. Application

Polyphenol level as measured by the present sensor ranged from 150.3 to 180.1 μM in tea leaves, 140.6 to 170.4 μM in alcoholic beverages and 1.4 to 1.6 μM in water samples (Table 2).

4. Conclusions

The covalent immobilization of polyphenol oxidase onto chitosan coated nylon membrane has resulted into improved analytical performance of polyphenol biosensor in terms of lower response time (15 s), lower limit of detection (0.2 μM), wider working range (0.2–400 μM) and higher storage stability (180 days at 4 °C) compared to other membrane based biosensors.

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